

# Cross Kingdom Functional Conservation of the Core Universally Conserved Threonylcarbamoyladenine tRNA Synthesis Enzymes

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Threonylcarbamoyladenine ( $t^6A$ ) is a universal modification located in the anticodon stem-loop of tRNAs. In yeast, both cytoplasmic and mitochondrial tRNAs are modified. The cytoplasmic  $t^6A$  synthesis pathway was elucidated and requires Sua5p, Kae1p, and four other KEOPS complex proteins. Recent *in vitro* work suggested that the mitochondrial  $t^6A$  machinery of *Saccharomyces cerevisiae* is composed of only two proteins, Sua5p and Qri7p, a member of the Kae1p/TsaD family (L. C. K. Wan et al., *Nucleic Acids Res.* 41:6332–6346, 2013, <http://dx.doi.org/10.1093/nar/gkt322>). Sua5p catalyzes the first step leading to the threonyl-carbamoyl-AMP intermediate (TC-AMP), while Qri7 transfers the threonyl-carbamoyl moiety from TC-AMP to tRNA to form  $t^6A$ . Qri7p localizes to the mitochondria, but Sua5p was reported to be cytoplasmic. We show that Sua5p is targeted to both the cytoplasm and the mitochondria through the use of alternative start sites. The import of Sua5p into the mitochondria is required for this organelle to be functional, since the TC-AMP intermediate produced by Sua5p in the cytoplasm is not transported into the mitochondria in sufficient amounts. This minimal  $t^6A$  pathway was characterized *in vitro* and, for the first time, *in vivo* by heterologous complementation studies in *Escherichia coli*. The data revealed a potential for TC-AMP channeling in the  $t^6A$  pathway, as the coexpression of Qri7p and Sua5p is required to complement the essentiality of the *E. coli tsaD* mutant. Our results firmly established that Qri7p and Sua5p constitute the mitochondrial pathway for the biosynthesis of  $t^6A$  and bring additional advancement in our understanding of the reaction mechanism.

As the central adaptors responsible for accurate decoding of mRNA, tRNA molecules are critical for cell survival. In order to ensure the accuracy of decoding, tRNAs harbor posttranscriptional modifications mainly in the anticodon stem loop (ASL) (1). N<sup>6</sup>-threonyl-carbamoyl-adenosine ( $t^6A$ ), a modification of adenosine located at position 37 next to the anticodon ( $t^6A_{37}$ ), is a highly conserved tRNA modification present in all domains of life. This modification is present in nearly all ANN-decoding tRNAs (one exception is tRNA<sup>Met</sup> in bacteria) (2). Like other modifications found at position 37, such as m<sup>1</sup>G, i<sup>6</sup>A, or ms<sup>2</sup>i<sup>6</sup>A modifications (3, 4),  $t^6A$  is important for translation fidelity because it stabilizes the ASL structure and enhances codon-anticodon interaction (5).

The enzymatic synthesis of  $t^6A$  has been studied *in vitro* and *in vivo* for more than 40 years (2, 6–9), but the synthesis pathways were only recently elucidated, revealing both a core set of enzymes and kingdom-specific variations (Fig. 1) (10, 11). Two universal protein families are involved in the biosynthesis of  $t^6A$ : TsaC/Sua5 and Kae1/Qri7/TsaD (12, 13). In *Escherichia coli*, *tsaC* (previously *yrdC*) and *tsaD* (previously *ygiD*) are essential for growth, and in *Saccharomyces cerevisiae*, *SUA5* (a *tsaC* homolog) and *KAE1* (a cytoplasmic member of the Kae1/Qri7/TsaD family) both are required for  $t^6A$  synthesis (12, 13). Kae1 is a member of the KEOPS complex (kinase, putative endopeptidase, and other proteins of small size) (14), also known as EKC (endopeptidase-like kinase chromatin-associated complex) (15). TsaC and TsaD from *E. coli*, along with bicarbonate, L-threonine, ATP, and tRNA, are not sufficient to form  $t^6A$  *in vitro*; TsaB (previously *YeaZ*) and TsaE (previously *YjeE*) also are required (10). This result was reproduced using the *Bacillus subtilis* orthologs, and it was shown that YwlC of *B. subtilis* (YwlC<sub>Bs</sub>; the ortholog of TsaC of *E. coli* [TsaC<sub>Ec</sub>]) con-

verts carbon dioxide (or bicarbonate), L-threonine, and ATP to the L-threonylcarbamoyl-AMP (TC-AMP) intermediate (16). TC-AMP then is converted to  $t^6A$  by YdiC<sub>Bs</sub> (TsaB<sub>Ec</sub>), YdiB<sub>Bs</sub> (TsaE<sub>Ec</sub>), and YdiE<sub>Bs</sub> (TsaD<sub>Ec</sub>) in the presence of tRNA (16). This work was extended to show that TC-AMP produced by TsaC or Sua5p from eukaryotic or archaeal origin can be converted to  $t^6A$ -modified tRNA by the *E. coli* TsaDEB enzymes, the *S. cerevisiae* KEOPS complex, or the *Pyrococcus abyssi* KEOPS complex (11). In short, the same enzyme family in all organisms, TsaC/Sua5, catalyzes the formation of TC-AMP. For the next step of transferring TC to tRNA, bacteria require TsaBDE, while archaea and eukaryotes use the KEOPS complex. Interestingly, although TsaBDE and KEOPS are functional analogues, only the TsaD/Kae1 proteins are shared between the two systems (Fig. 1), suggesting this protein was part of the ancestral  $t^6A$  synthesis core present in the last universal common ancestor (LUCA) (17). This hypothesis was strengthened by the demonstration that in *S. cerevisiae* a minimal system comprised of just Sua5p and the Kae1/TsaD mitochondrial ortholog Qri7p is sufficient to synthesize  $t^6A$  *in vitro* (17).

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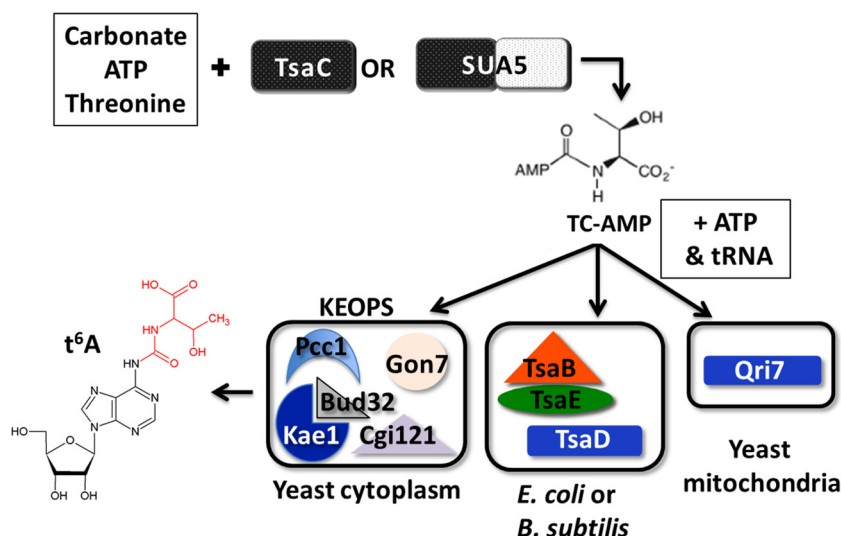
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**FIG 1** Diversity in the biosynthesis pathway of the universal tRNA modification t<sup>6</sup>A. The formation of TC-AMP is catalyzed by the same enzyme family in all organisms (TsaC/Sua5). Sua5p contains a TsaC domain plus an additional C-terminal domain, called Sua5, that has yet to be characterized. To transfer threonylcarbamoyl (TC) to tRNA, bacteria require TsaBDE, while archaea and eukaryotes use the KEOPS complex (composed of Kae1, Bud32, Cgi121, and Pcc1 proteins; Gon7 is found exclusively in fungi).

In *S. cerevisiae*, mitochondrial DNA (mtDNA) encodes all of the required mitochondrial tRNAs, and they have been shown to contain t<sup>6</sup>A (18). Qri7p has been shown to localize to the mitochondria in several organisms (19, 20), and *qri7Δ* in *S. cerevisiae* does not grow on glycerol, indicating a mitochondrial defect (19, 21). Also, when the mitochondrial targeting sequence of Qri7p is deleted, Qri7p can functionally replace Kae1p in t<sup>6</sup>A synthesis in the cytoplasm of *S. cerevisiae* cells (12). Therefore, it was proposed that the mitochondrial t<sup>6</sup>A system is minimal and composed of only Sua5p and Qri7p (11, 17).

Several questions remain to be answered to complete the understanding of the yeast mitochondrial pathway. First, it is not clear if TC-AMP, the product of the Sua5p-catalyzed reaction, is transported to the mitochondria or if the Sua5 protein itself is imported into this organelle. Global subcellular localization studies in *S. cerevisiae* reported Sua5p to localize to the cytoplasm (20, 22). Second, why can Qri7, unlike its bacterial counterparts, function without the TsaB and TsaE proteins? A few bacteria, mainly intracellular pathogens such as *Mycoplasma*, have lost *tsaB* and *tsaE* (23). It is not clear why these genes were kept in the majority of sequenced bacteria to date. Third, TC-AMP (produced by TsaC or Sua5p) is used indifferently by the TsaBDE, KEOPS, or Qri7p systems *in vitro*, but is this also the case *in vivo*? Indeed, as the TC-AMP intermediate is highly unstable (16), it is likely that the t<sup>6</sup>A synthesis *in vivo* requires a protection mechanism for TC-AMP.

In this work, we validate the hypothesis that Sua5p participates in mitochondrial t<sup>6</sup>A synthesis by showing that Sua5p localizes to both the cytoplasm and the mitochondria through an alternative translation start site and explore the possibility of a TC-AMP protection mechanism via channeling through heterologous complementation studies.

## MATERIALS AND METHODS

**Strains and growth conditions.** Yeast strains were grown on yeast extract-peptone-dextrose (YPD; Difco Laboratories) at 30°C. Synthetic minimal

media, with or without agar and with or without dropout (lacking uracil [–ura], leucine, [–leu], and/or histidine [–his]), were purchased from Clontech (Palo Alto, CA) and prepared as recommended by the manufacturer. Glucose (Glu; 2%, wt/vol), glycerol (Gly; 4%, wt/vol), 5-fluoroorotic acid (5-FOA; 0.1%, wt/vol), and G418 (300 μg/ml) were used when appropriate. Yeast transformations were carried out using frozen competent cells as described previously (24), with plating onto the appropriate media. The *sua5::KANMX4* strain VDC9100 was created by first PCR amplifying the *sua5::KanMX4* locus from the *sua5::KANMX4/SUA5* diploid obtained from Euroscarf with oligonucleotides flanking the allele. The PCR product then was transformed via electroporation into wild-type BY4742 containing pBN204 (*SUA5* on a *URA3* plasmid) (13). Transformants were selected on SD–ura plates containing G418. After passaging on uracil-containing media, the strain was cured of pBN204 by counterselection with 5-FOA. Disruption of *SUA5* was confirmed by PCR using oligonucleotides annealing inside and outside the locus, as well as inside *KANMX4*. VDC9100 was confirmed to be devoid of t<sup>6</sup>A using the high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses listed below.

*E. coli* strains were grown in LB (1%, wt/vol, tryptone, 0.5%, wt/vol, yeast extract, and 1%, wt/vol, salt; 1.5%, wt/vol, agar was added for plates) at 37°C unless otherwise stated. When necessary, LB was supplemented with kanamycin (Km; 50 μg/ml), ampicillin (Ap; 100 μg/ml), chloramphenicol (Cm; 35 μg/ml), anhydrotetracycline (aTc; 50 ng/ml), and arabinose (Ara; 0.2%, wt/vol). Plasmids were transformed into *E. coli* via electroporation. For drop dilution assays, *E. coli* cells were grown to saturation overnight in LB supplemented with aTc with the appropriate antibiotics and diluted in 10-fold increments with or without a prior wash step to remove any aTc left in the culture media. Ten μl of each dilution then was spotted onto the appropriate media. For halo plates, cultures were grown to saturation overnight in LB supplemented with aTc with the appropriate antibiotics and then diluted 500 times. Five ml of the diluted culture was added to plates containing 25 ml of agar media, and the excess bacterial suspension was immediately removed. Plates were dried, and a 10-μl drop of aTc was added on the surface of the plate as shown in Fig. S3 in the supplemental material.

**Plasmid construction for protein expression.** All plasmids constructed for protein expression (Table 1) and sequences of all primers and restriction sites used for cloning are listed in Table S1 in the supplemental

TABLE 1 Strains and plasmids used in this study

Strain or plasmid name	Relevant characteristic(s)	Reference or source
<b>Yeast</b>		
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	39
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	39
YGL169w::GFP	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SUA5::GFP</i>	22
YDL104c::GFP	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 QRI7::GFP</i>	22
VDC9054	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sua5::kanMX4</i> (pBN204)	This study
VDC9100	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sua5::kanMX4</i>	This study
<b><i>E. coli</i></b>		
DH5α	F <sup>−</sup> <i>gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(r<sub>B</sub><sup>−</sup>, m<sub>B</sub><sup>−</sup>) supE44 ara-14 galK2 lacY1 proA2 rpsL20(Sm<sup>r</sup>) xyl-5 λ<sup>−</sup> leu mtl1</i>	Invitrogen
BW25113	F <sup>−</sup> <i>Δ(araD-araB)567 ΔlacZ4787(::rrnB-3) λ<sup>−</sup> rph-1 Δ(rhaD-rhaB)568 hsdR514</i>	<i>E. coli</i> genetic stock center
VDC5789	BW25113 containing <i>knRExTETrbs::tsaD<sub>Ec</sub></i>	12
VDC5684	BW25113 containing <i>knRExTETrbs::tsaC<sub>Ec</sub></i>	This study
VDC5806	VDC5789(pBAD24)	This study
VDC5732	VDC5684(pBAD24)	This study
VDC6116	VDC5684(pBY265.9)	This study
VDC6120	VDC5684(pBY270.2)	This study
VDC6126	VDC5789(pBY265.9)	This study
VDC6130	VDC5789(pBY270.2)	This study
VDC5926	VDC5789(pBY245.1)	This study
<b>Plasmids</b>		
pBAD24	<i>bla</i> , <i>araC</i> , P <sub>BAD</sub>	26
pBAD33	<i>cat</i> , <i>araC</i> , P <sub>BAD</sub>	26
pUC19	General cloning vector	40
pRS313	Yeast shuttle vector; <i>HIS3</i>	41
pBN204	pYES:: <i>SUA5</i> ; <i>URA3</i>	13
pYX122-mtGFP	Mitochondrial targeting GFP; <i>HIS3</i>	25
pYX142-mtRFP	Mitochondrial targeting RFP; <i>LEU2</i>	A. Delahodde
pPCT004	pYX122-mtGFP fused to first 70 bp of <i>SUA5</i> cloned as EcoRI/BamHI fragment	This study
pPCT006	pYX122-mtGFP fused to 5'-truncated <i>SUA5</i> cloned as EcoRI/BamHI fragment	This study
pPCT017	pUC19:: <i>SUA5</i> cloned as EcoRV fragment	This study
pPCT019	pUC19:: <i>SUA5</i> (M10L) cloned as EcoRV fragment	This study
pPCT025	pYX122-mtGFP:: <i>SUA5</i> subcloned from pPCT017 as EcoRI/BamHI fragment	This study
pPCT030	pYX122-mtGFP:: <i>SUA5</i> (M10L) subcloned from pPCT019 as EcoRI/BamHI fragment	This study
pPCT036	pYX122-mtGFP fused to first 70 bp of <i>SUA5</i> (M10L) cloned as EcoRI/BamHI fragment	This study
pPCT066	<i>Sua5</i> <sub>(10-426)</sub> subcloned from pPCT006 as EcoRI/SmaI fragment into pRS313 treated with EcoRI/EcoRV	This study
pPCT070	<i>GAL</i> promoter subcloned from pBY137 as SpeI/PvuII fragment into pPCT066 digested with XbaI/SmaI	This study
pPCT074	<i>GAL::tsaC</i> subcloned from pBY135 as SpeI/PmeI fragment into pRS313 digested with SpeI and EcoRV	This study
pPCT075	<i>GAL::ywlC</i> subcloned from pBY137 as SpeI/PmeI fragment into pRS313 digested with SpeI and EcoRV	This study
pPCT076	Site-directed mutagenesis of pBY176 to change the first AUG of <i>SUA5</i> to CUG	This study
pPCT078	Site-directed mutagenesis of pBY176 to change the second AUG of <i>SUA5</i> to CUG	This study
pBY130	pBAD24:: <i>SUA5</i>	13
pBY135	pYesDEST52:: <i>tsaC<sub>Ec</sub></i> ; <i>bla</i> , <i>URA3</i>	13
pBY137	pYesDEST52:: <i>ywlC<sub>Bs</sub></i> ; <i>bla</i> , <i>URA3</i>	13
pBY176	pRS313:: <i>SUA5<sub>Sc</sub></i> ; <i>bla</i> , <i>URA3</i>	13
pBY265.9	pBY130:: <i>QRI7</i> (primer pair 18/19)	This study
pBY270.2	pBAD24:: <i>QRI7</i> (primer pair 18/19)	This study
pBY245.1	pBAD24:: <i>tsaD</i> (primer pair 16/17)	This study
pBY343.2	pBAD33':: <i>tsaD</i> (primer pair 22/23); pBAD33' is pBAD33 cut with EcoRV and SmaI, partially removing <i>araC</i> and the P <sub>BAD</sub> promoter; <i>tsaD</i> is constitutively expressed from P <sub>BAD</sub> promoter brought in by the forward primer	This study
pBY344.2	pBAD33':: <i>QRI7</i> (primer pair 20/21)	This study
pBY346.2	pBY344.2:: <i>rbsSUA5Met1</i> (primer pair 24/26); <i>SUA5</i> is cloned from the first methionine	This study
pBY347.6	pBY344.2:: <i>rbsSUA5Met10</i> (primer pair 25/26); <i>SUA5</i> is cloned from the second methionine	This study

material. All plasmid constructs were confirmed by sequencing. The cloning procedure for protein purification of *S. cerevisiae* Sua5 (Sua5<sub>sc</sub>) was described elsewhere (11). *QRI7* was amplified by PCR using genomic DNA from *S. cerevisiae* strain S288C (ATCC) and appropriate oligonucleotides (see Table S1). The first 90 nucleotides of *QRI7*, encoding the mitochondrial targeting sequence, were deleted. The gene carried a hexahistidine tag at the 3' end and subsequently was cloned into pET28a expression vector (Novagen) via NcoI and NotI restriction sites.

**Plasmid construction for yeast *in vivo* assays.** Plasmid construction for microscopy was based on the yeast mitochondrion-targeting green fluorescent protein (GFP) plasmid, pYX122-mtGFP (25). pYX122-mtGFP was digested with EcoRI and BamHI to remove the preSu9 mitochondrion-targeting sequence. *SUA5* from BY4742 was PCR amplified with oligonucleotides listed in Table S1 in the supplemental material to create the various forms of Sua5-GFP. PCR products were digested with EcoRI and BamHI and ligated using T4 DNA ligase (NEB) into the similarly digested pYX122-mtGFP. Plasmids for complementation assays listed in Table 1 were created by subcloning components from previously described plasmids. Site-directed mutagenesis was performed using the QuikChange lightning mutagenesis kit (Agilent, Santa Clara, CA) by following the manufacturer's recommendations.

**Plasmid construction for *E. coli in vivo* assays.** *E. coli tsaD* (NP\_417536) was amplified by PCR from genomic DNA prepared from *E. coli* K-12 MG1655 with oligonucleotides listed in Table S1 in the supplemental material. The yeast *SUA5* (NP\_011346) and *QRI7* (NP\_010179) genes were amplified from *S. cerevisiae* BY4741 genomic DNA with oligonucleotides listed in Table S1. PCR fragments were digested with appropriate restriction enzymes before cloning into pBAD or pBAD-derived vectors (26). *QRI7* was cloned without its putative mitochondrion-targeting sequence (corresponding to the first 30 amino acids). The ribosomal binding site and/or restriction sites were added to primer sequences for cloning purposes as described in Table S1. *SUA5* was cloned from either the first (M<sub>1</sub>) or second methionine (M<sub>10</sub>).

**Microscopy.** To determine the localization of Sua5-GFP, yeast were cotransformed with the mitochondrial marker pYX142-RFP and one of the Sua5-GFP constructs, and transformants were selected on SD–his–leu plates. For microscopy, cells were grown to mid-log phase in dropout media containing 1% glucose and 3% glycerol. Cells were washed once with water prior to analysis at 100× magnification on a Leica DM IRE2 confocal microscope using MetaMorph microscopy automation and image analysis software (Molecular Devices, Sunnyvale, CA). Deconvolution and pseudocoloring of images was performed using MetaMorph, and final figures were constructed using Photoshop CS6 (Adobe).

**Recombinant protein expression and purification.** The expression and purification of recombinant Sua5p from *S. cerevisiae* and TsaC, TsaD, TsaB, and TsaE from *E. coli* was described previously (11). Recombinant Qri7p was expressed in *E. coli* Rosetta2 (DE3) pLysS (Novagen). Protein overexpression was induced in overnight express instant TB medium (Novagen) supplemented with 10% glycerol. Cells were collected by centrifugation, suspended in lysis buffer (LBf; 50 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol, 0.01% [vol/vol] Triton X-100, 10 mM imidazole, protease inhibitor cocktail [Roche]), and sonicated on ice. His-tagged proteins from the soluble fraction were purified by gravity-flow chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) resin column (Qia-Gen) according to the manufacturer's recommendations. Fractions of interest were pooled, concentrated, and injected on a Superdex 200 column (GE Healthcare). Fractions containing pure proteins were concentrated and stored at –80°C in storage buffer (SB; 50 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol).

**Yeast growth assays.** Growth curves were performed using a Bioscreen C MBR (Oy Growth Curves AB Ltd., Finland) at 30°C with maximum shaking. A 250-μl culture was used in each well, and 5 biological replicates were used for each condition. Yeast cultures were grown in SD–his to saturation, normalized to an optical density at 600 nm (OD<sub>600</sub>) of 1, and diluted 200 times in SD–his before being loaded on the Bio-

screen. The growth curves presented are averages from 5 biological replicates. Significance was determined using a 2-way analysis of variance (ANOVA) and Fisher's least significant differences (LSD) in Prism 6 (GraphPad).

**Preparation of bulk tRNA and detection of t<sup>6</sup>A.** Bulk tRNA was prepared by double phenol-chloroform extraction, and nucleoside preparations were prepared as previously described (13). All tRNA extractions were performed in triplicate from independent cultures. t<sup>6</sup>A was detected by HPLC as described previously (27) and LC-MS/MS as described in reference 13. The MS/MS fragmentation data and a synthesized t<sup>6</sup>A standard provided by Darrell Davis (University of Utah) were used to confirm the presence of t<sup>6</sup>A.

**tRNA production and purification.** *E. coli* tRNA<sup>Asn(GUU)</sup> and tRNA<sup>Lys(UUU)</sup> were overexpressed in *E. coli* XL1-Blue cells (Agilent Technologies) and purified as described previously (11). For purification, RNAs were loaded on an 8 M urea, 12% polyacrylamide gel, and the band corresponding to overexpressed tRNA was cut out. tRNA was eluted from gel in elution buffer (EB; 0.1% SDS, 0.5 M ammonium acetate [NH<sub>4</sub>Ac], 10 mM MgAc, 1 mM EDTA), precipitated, and suspended in water. To favor correct folding of tRNAs, the solution was heated at 70°C, cooled slowly to room temperature, and stored at –80°C.

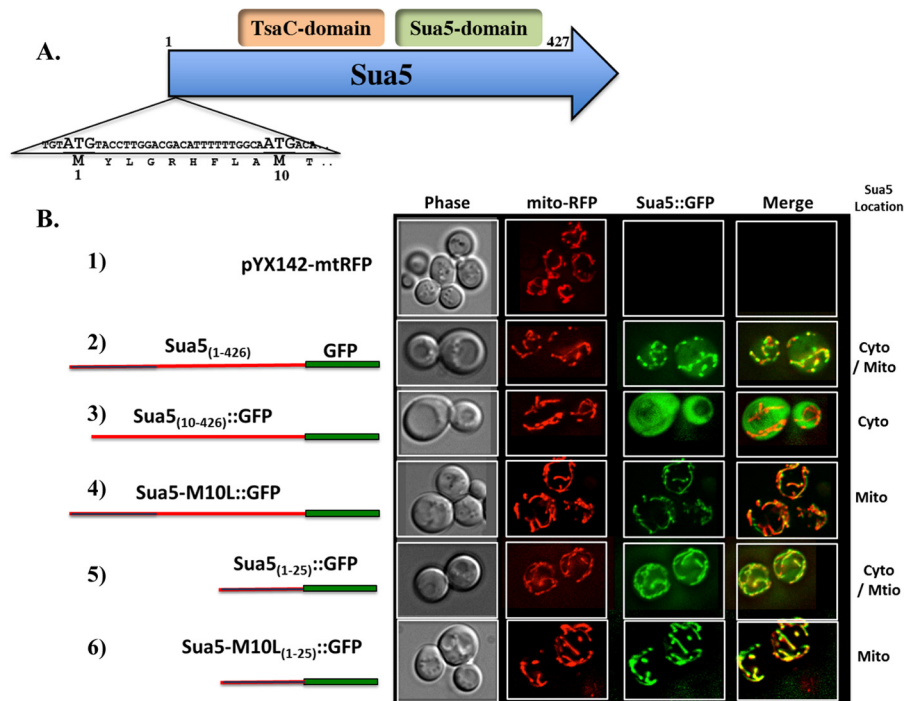
***In vitro* assay for the synthesis of t<sup>6</sup>A-modified tRNA.** The *in vitro* assay was performed essentially as described previously (11). The reaction was performed in a final volume of 50 μl containing 4 μM Sua5p and 5 μM Qri7p, 4.8 μM tRNA, and 18.2 μM [<sup>14</sup>C]L-threonine (0.05 mCi, 55 Ci/mol; Hartmann Analytic) in reaction buffer (RB). After 40 min of incubation at 32°C, macromolecules were precipitated by the addition of 1 ml of trichloroacetic acid 15% (TCA) and incubated on ice for 1 h. Precipitated material was applied on prewet glass microfiber GF/F filters (Whatman) using a vacuum apparatus (Millipore). Filters were washed with 3 ml of 5% TCA and 3 ml of 95% ethanol (EtOH) and dried. Radioactivity was recorded with a liquid scintillation analyzer (Packard) as average counts per minute (cpm) for 2 min. The amount of incorporated threonine was calculated from a standard curve obtained with different concentrations of [<sup>14</sup>C]L-threonine spotted on filters (1 pmol = 102.8 cpm). The yield of the reaction was calculated as [cpm (assay) – cpm (background)]/102.8]/(pmol tRNA) × 100.

## RESULTS

**Sua5p is targeted to the cytoplasm and to the mitochondria by alternate in-frame AUG start sites.** *In vitro*, Qri7p and Sua5p can produce t<sup>6</sup>A without any additional proteins, even when separated by a 2-kDa molecular size cutoff membrane, although noticeably more t<sup>6</sup>A is produced when Qri7p and Sua5p are not separated (the precise increase is difficult to determine from Fig. 3A of Wan et al. [17]). Close examination of the *SUA5* open reading frame (ORF) revealed two in-frame AUG codons located in the first 28 nucleotides (Fig. 2A), and we postulated that, *in vivo*, Sua5p was functional in both the cytoplasm and mitochondria, as no *SUA5* homolog is encoded by the mtDNA. The mitochondrial targeting prediction software iPSORT (28) predicted a mitochondrion-targeting sequence only for the isoform that started at the first AUG (here referred to as M<sub>1</sub>, for methionine at position 1) and did not predict a mitochondrial targeting sequence for the second AUG (M<sub>10</sub>, methionine at position 10 of Sua5). This suggested that a long form of Sua5p translated from the first AUG was a mitochondrion-targeted protein, whereas a short form of Sua5p, translated from the second AUG, would remain cytoplasmic.

To test these predictions, we performed fluorescence microscopy of a chromosomally encoded Sua5-GFP fusion, and as a control, we tested Kae1-GFP and Qri7-GFP. Because of the low abundance of each of these proteins (estimated at less than 600 molecules per cell for Sua5p [20]), the signal was weak and pro-





**FIG 2** Subcellular localization of Sua5p. (A) The *SUA5* ORF contains two in-frame AUG codons located in the first 28 nucleotides. iPSORT (28) predicts Sua5p translated from the first AUG would target to the mitochondria. The TsaC-like domain and Sua5 domain are indicated to scale. (B) Sua5::GFP localization using confocal microscopy at 100 $\times$  magnification. Wild-type BY4741 was transformed with plasmids expressing RFP targeted to the mitochondria or Sua5::GFP fusions. Row 1, control. Row 2, RFP fused to Fo-ATPase mitochondrial targeting sequence. Full-length, wild-type Sua5::GFP indicates cytoplasmic and mitochondrial targeting of Sua5p. Colocalization of RFP and GFP in mitochondria is indicated as yellow in the merged image. Row 3, residues 10 to 426 of Sua5 fused to GFP [Sua5<sub>(10-426)</sub>::GFP] only targets to the cytoplasm. Row 4, substitution of M10L in full-length Sua5 (Sua5-M10L::GFP) enhances localization to the mitochondria. Row 5, the first 25 residues of Sua5p [Sua5<sub>(1-25)</sub>] is sufficient for dual targeting. GFP is located in both the cytoplasm and mitochondria. (6) Sua5-M10L<sub>(1-25)</sub>::GFP only targets to the mitochondria.

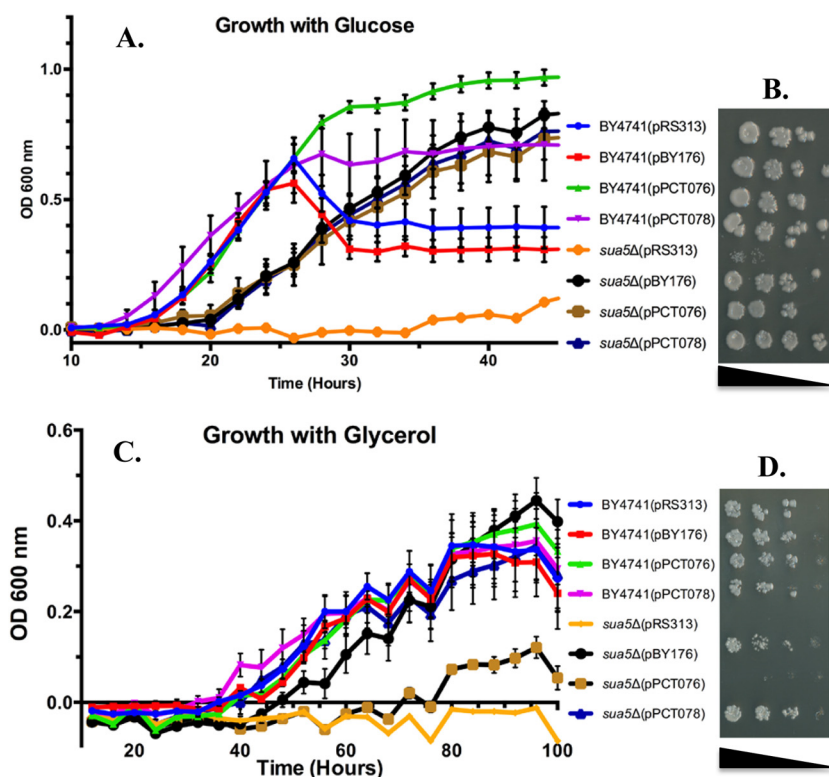
duced images that were greatly overexposed, but they did indicate the mitochondrial localization of Sua5p (data not shown). To increase the level of Sua5p in the cell, we constructed Sua5-GFP fusions under the control of the constitutive triosephosphate isomerase (TPI) promoter in the ARS/CEN vector pYX122-mtGFP (25). The Su9 mitochondrial targeting sequence in pYX122-mtGFP was replaced with *SUA5* lacking a stop codon, creating plasmid pPCT025. Wild-type *S. cerevisiae* BY4741 was cotransformed with pPCT025 and pYX142-mtRFP (to specifically label mitochondria with red fluorescent protein [a gift from Agnès Delahodde]), and transformants were examined by 100 $\times$  confocal microscopy. The experiment revealed uniform distribution of GFP in the cytoplasm as well as in foci of GFP in the mitochondria colocalizing with mitochondrion-targeted red fluorescent protein (RFP) (Fig. 2B, row 2). We next examined a 5'-truncated form of Sua5p created by cloning Sua5<sub>(10-426)</sub> into pYX122-mtGFP, giving pPCT006. Sua5p starting at the second Met (M<sub>10</sub>) localized exclusively to the cytoplasm (Fig. 2B, row 3). Additionally, we created a point mutation in *SUA5* altering the second AUG to CUG (M10L) and cloned *SUA5*<sub>(M10L)</sub> into pYX122-mtGFP, creating pPCT030. As seen in Fig. 2B, row 4, the M10L substitution of Sua5p enhances the localization to the mitochondria, eliminating Sua5-GFP in the cytoplasm. To confirm that the N terminus of Sua5p was responsible for targeting GFP to the mitochondria, we cloned the first 70 nucleotides of *SUA5* and *SUA5*<sub>(M10L)</sub> into pYX122-mtGFP, creating pPCT004 and pPCT036, respectively. As seen in Fig. 2B, rows 5 and 6, the first 70 nucleotides of *SUA5* dual-localized GFP to the

cytoplasm and the mitochondria, but when the second AUG is mutated to CUG, GFP localized only to the mitochondria, supporting the conclusion that Sua5p can target the mitochondria.

The localization and function of Sua5-GFP was confirmed by cotransforming VDC9100 (*sua5* $\Delta$ ) with pYX142-RFP and each of the Sua5-GFP plasmids. The targeting of Sua5-GFP in *sua5* $\Delta$  mimics the results seen in wild-type BY4741. Full-length Sua5-GFP targeted to both the cytoplasm and mitochondria. Sua5<sub>(10-426)</sub>-GFP targeted exclusively to the cytoplasm, while Sua5<sub>(M10L)</sub>-GFP targeted exclusively to the mitochondria (see Fig. S1 in the supplemental material).

**TC-AMP produced in the cytoplasm is not sufficient for mitochondrial function.** To test if TC-AMP could be shuttled from the cytoplasm to the mitochondria, a series of plasmids were constructed encoding Sua5p targeted to the cytoplasm only, TsaC from *E. coli*, and Sua5 (YwlC) from *B. subtilis*. To mimic the naturally low expression of *SUA5*, P<sub>GAL</sub> fusions in the low-copy-number CEN/ARS plasmid pRS313 were constructed. P<sub>GAL</sub> is known to be leaky, even in the presence of glucose (29). Therefore, Sua5<sub>(10-426)</sub> was cloned along with P<sub>GAL</sub> from pYesDEST52 into pRS313 (creating pPCT070), allowing Sua5<sub>(10-426)</sub> (targeted to the cytoplasm) to be expressed at low levels in the absence of the galactose inducer. This also eliminates possible interfering growth on galactose when testing growth with glycerol as the carbon source. P<sub>GAL</sub>::*tsaC*<sub>Ec</sub> (pPCT074), P<sub>GAL</sub>::*ywlC*<sub>Bs</sub> (pPCT075), and P<sub>GAL</sub>::*SUA5*<sub>Sc</sub> (pBY176) were constructed using the same plasmid, pRS313, as the backbone.

Yeast strains with mutations in *SUA5* show slow growth in



**FIG 3** Complementation of *sua5Δ*. Growth curves performed in Bioscreen C. Points represent the averages from five biological replicates. Error bars represent the standard errors of the means. pBY176 contains *SUA5* plus 200 nt of sequence upstream cloned into pRS313. pPCT076 contains an M1L mutation of *SUA5*. pPCT078 contains an M10L mutation of *SUA5*. (A) Complementation of *sua5Δ* on 2% glucose. (B) Growth on 2% glucose-containing plates at day 4. Five-microliter drops of 10-fold dilutions beginning at an OD<sub>600</sub> of 0.1. (C) Complementation of *sua5Δ* on 2% glycerol. (D) Same as panel B, except growth on 2% glycerol-containing plates at day 10 was examined.

glucose-containing media and cannot utilize the nonfermentable sugar alcohol glycerol as a sole carbon source (13, 30). As shown in Fig. S2 in the supplemental material, supplying P<sub>GAL</sub>::*SUA5*<sub>(10-426)</sub>, P<sub>GAL</sub>::*tsaC<sub>Ec</sub>*, P<sub>GAL</sub>::*ywlC<sub>Bs</sub>*, or P<sub>GAL</sub>::*SUA5<sub>Sc</sub>* in trans suppressed the growth defect of the *sua5Δ* strain using glucose as a carbon source, implying the corresponding proteins were functional in the cytoplasm. However, only P<sub>GAL</sub>::*SUA5<sub>Sc</sub>* restored growth on glycerol, albeit with a longer lag phase.

These results suggest that these proteins produced enough TC-AMP in the cytoplasm to suppress the glucose growth defect and that TC-AMP was not being shuttled to the mitochondria, as seen by the inability of P<sub>GAL</sub>::*SUA5*<sub>(10-426)</sub>, P<sub>GAL</sub>::*tsaC<sub>Ec</sub>* or P<sub>GAL</sub>::*ywlC<sub>Bs</sub>* variants to grow on glycerol.

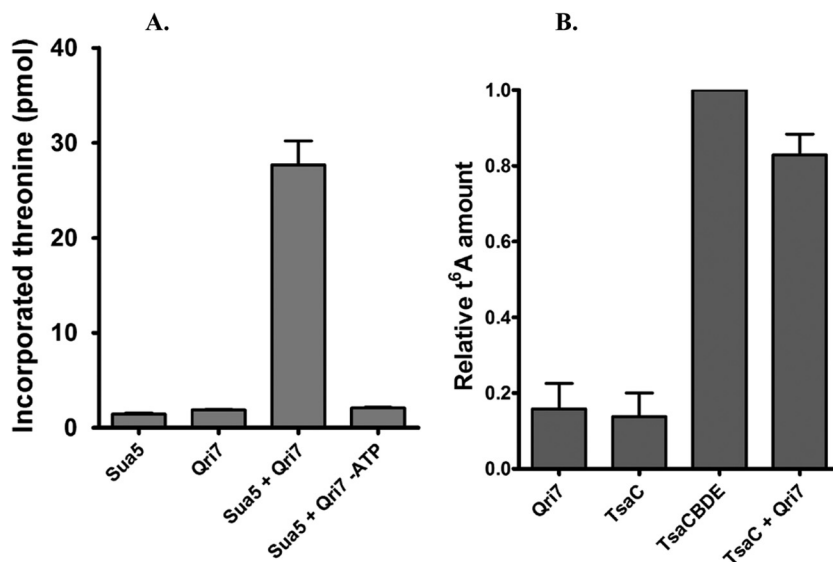
**Sua5p must be targeted to the mitochondria to grow on glycerol.** To directly test if mitochondrion-targeting Sua5p was required for mitochondrial function, a series of plasmids were prepared that use the native *SUA5* promoter to drive constructs targeting Sua5p to the cytoplasm, mitochondria, or both. Site-directed mutagenesis was performed on pBY176 (*SUA5* plus 200 nt upstream sequence cloned into the low-copy-number plasmid pRS313) to mutate the first methionine to leucine (M1L), creating pPCT076, or the second methionine to leucine (M10L), creating pPCT078. All three *SUA5*-containing plasmids, as well as the parental pRS313, were transformed in BY4741 and the *sua5Δ* mutant with selection on SD–his agar.

Mitochondrial function was assayed by testing for growth on the nonfermentable carbon source glycerol. The expression of any

of the *SUA5*-containing plasmids had no effect on the growth of BY4741 using glucose or glycerol as a carbon source (Fig. 3). The expression of *SUA5* with the first AUG or the second AUG mutated complemented the slow growth of *sua5Δ* to the same levels as wild-type *SUA5* when glucose was used as a carbon source (Fig. 3A and B). However, when glycerol was used as a carbon source, expression of wild-type *SUA5* and of *SUA5* with the second AUG mutated [encoding Sua5<sub>(M10L)</sub>] complemented the lack of growth of the *sua5Δ* mutant (Fig. 3C and D). The expression of *SUA5* with a mutation in the first AUG did not allow complementation.

This complementation assay corroborates the GFP localization data presented earlier. Sua5p is dual targeted to both the cytoplasm and the mitochondria through the use of alternative translational starts, and translation from the first AUG is required for localization to the mitochondria.

**In vitro, Qri7p can function with either Sua5p or TsaC<sub>Ec</sub>.** Wan et al. showed that Qri7p and Sua5p from *S. cerevisiae* are necessary and sufficient to synthesize t<sup>6</sup>A in vitro, and we reproduced this result using a different assay (10, 11) (Fig. 4A). The assay measures the incorporation of radioactive threonine into TCA-precipitated material that corresponds to the formation of t<sup>6</sup>A-modified tRNA (11). As shown in Fig. 4A, significant synthesis of t<sup>6</sup>A was obtained in an ATP-dependent manner only when both Sua5p and Qri7p were present in the reaction mixture. In contrast, only background signal levels were measured using Sua5p or Qri7p alone, indicating that both are necessary for the reaction to take place. We next asked if a heterologous system



**FIG 4** *In vitro* synthesis of t<sup>6</sup>A. (A) Qri7 and Sua5 proteins from *S. cerevisiae* catalyze the formation of t<sup>6</sup>A in ATP-dependent fashion. The reaction mixture contained 4  $\mu$ M Sua5p and 5  $\mu$ M Qri7p, 4.8  $\mu$ M *E. coli* tRNA<sup>Lys</sup><sub>UUU</sub>, and 18.2  $\mu$ M [<sup>14</sup>C]L-threonine in reaction buffer (RB). After 40 min of incubation at 32°C, macromolecules were precipitated with trichloroacetic acid, and radioactivity contained in tRNA was recorded using a liquid scintillation analyzer. Error bars indicate standard deviations of the means obtained from at least three independent experiments. (B) Heterologous system composed of TsaC and Qri7p is functional *in vitro*. Each reaction mixture contained 3  $\mu$ M proteins, indicated under the x axis, 4.8  $\mu$ M *E. coli* tRNA<sup>Asn</sup><sub>GUU</sub>, and 18.2  $\mu$ M [<sup>14</sup>C]L-threonine in reaction buffer (RB). After 35 min of incubation at 37°C, macromolecules were precipitated with trichloroacetic acid. Radioactivity contained in the tRNA was recorded using a liquid scintillation analyzer. Error bars indicate standard deviations of the means obtained from at least three independent experiments.

composed of TsaC<sub>Ec</sub>, the bacterial homolog of Sua5p, and Qri7p could catalyze t<sup>6</sup>A synthesis *in vitro*. As shown in Fig. 4B, TsaC and Qri7p result in about 83% modified tRNA compared to the TsaCBDE control, indicating that the Qri7/TsaC<sub>Ec</sub> combination is functional *in vitro*.

***In vivo*, Qri7p requires Sua5p and does not function with TsaC<sub>Ec</sub>.** Surprisingly, in contrast to our *in vitro* results, we found that expression of Qri7p alone failed to complement the essential phenotype of an *E. coli* strain not expressing *tsaD*. As shown in Fig. 5A, the *E. coli* P<sub>TET</sub>::*tsaD* strain transformed with pBAD24 derivatives expressing *QRI7* (pBY270.2) did not grow in the absence of aTc (required for induction of P<sub>TET</sub>) and in the presence of arabinose. When *QRI7* and *SUA5* were expressed from an operon under the P<sub>BAD</sub> promoter (pBY265.9) (a diagram of the genetic constructs can be found in Fig. 5B), partial complementation (reduced growth and smaller colonies compared to complementation with TsaD) was observed (Fig. 5A), suggesting that Qri7p and TsaC are not able to function together *in vivo*, while Qri7p and Sua5p are at least able to sustain partial growth. The same plasmid expressing *QRI7* and *SUA5* was transformed into the P<sub>TET</sub>::*tsaC* strain, and we allowed it to grow in the absence of aTc, establishing that *SUA5* is functional in this setup. Interestingly, complementation was more robust in the absence of both aTc and arabinose in the strain expressing the yeast *SUA5* and *QRI7* genes compared to the strain expressing the *E. coli* *tsaD* gene.

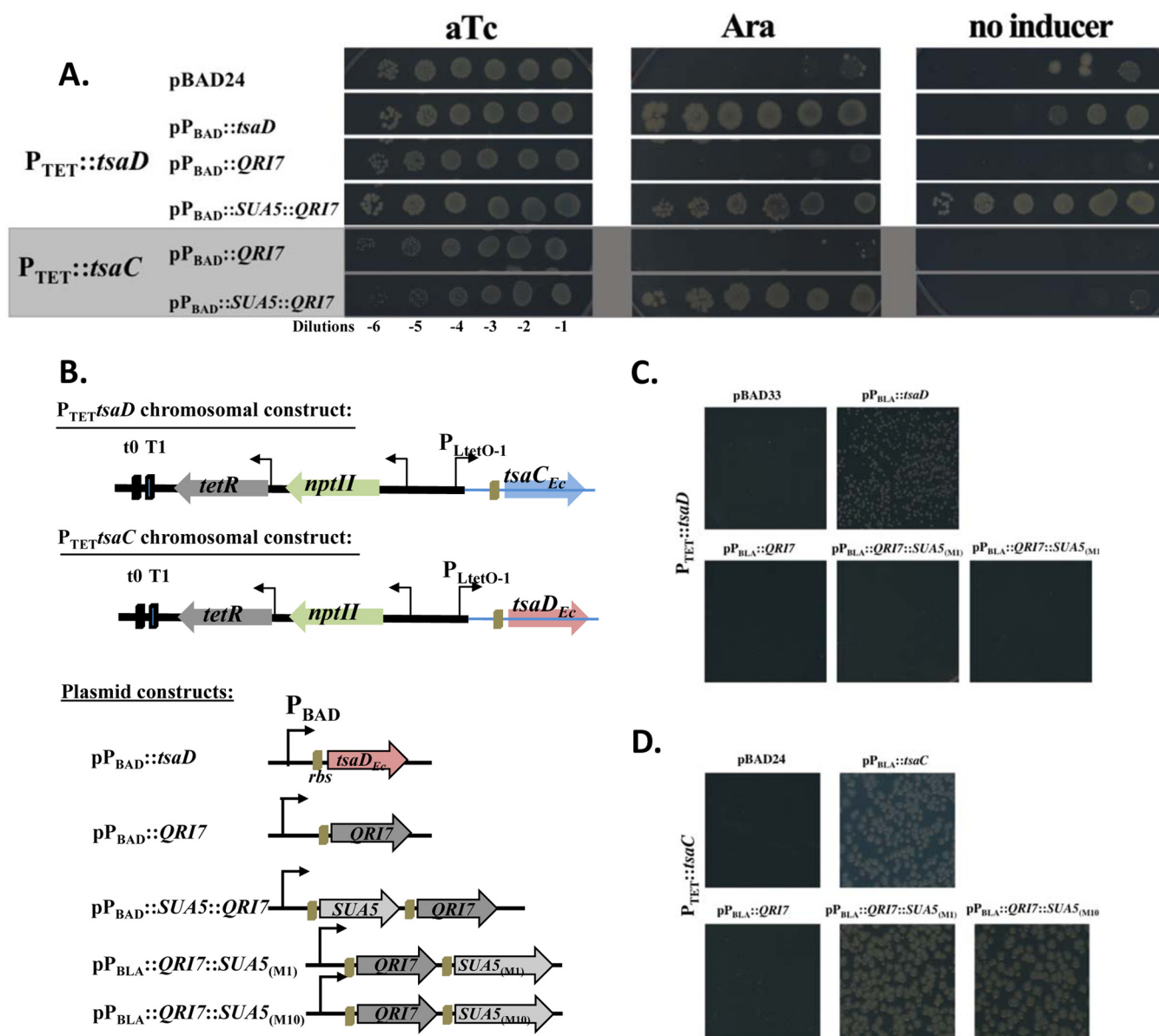
Similar results were obtained with halo assays, where diluted cultures were spread across plates and a 10- $\mu$ l drop of aTc was added at the center on the surface of the plate (see Fig. S3 in the supplemental material). The aTc diffuses through the plate and provides a gradient for induction of P<sub>TET</sub>. Partial complementation is observed only when both *QRI7* and *SUA5* are expressed as an operon under P<sub>BAD</sub>, and in the halo test, suppressors clearly appear in the zone with lower levels of aTc in the strain that ex-

press the yeast genes. These suppressors do not appear when the *E. coli* *tsaD* gene is expressed or with empty vector (see Fig. S3; suppressors are seen in the inset). Additionally, a low level of complementation was seen in the presence of Qri7p alone under conditions where a small amount of the chromosomal *E. coli* *tsaD* gene was produced. The diameter of halos from the aTc-dependent growth was slightly larger in the P<sub>TET</sub>::*tsaD* strain expressing Qri7p than in the empty vector control (see Fig. S3).

The ratio of Qri7p to Sua5p seems to be critical in allowing for the complementation of the P<sub>TET</sub>::*tsaD* strain, as expressing *QRI7* and *SUA5* in a lower-copy-number plasmid (P15a ori) under a constitutive promoter (P<sub>BLA</sub>) did not allow growth in the P<sub>TET</sub>::*tsaD* strain in the absence of aTc (Fig. 5C) when it did even in the absence of induction in the high-copy-number plasmid pBAD24 (ColE1 ori) (Fig. 5A). Expressing *E. coli* *tsaD* in the same plasmid (pBY343.2) did allow growth without aTc, establishing that the P<sub>BLA</sub> promoter is functional (Fig. 5C). To ensure *SUA5*<sub>(M1)</sub> and *SUA5*<sub>(M10)</sub> were functional in *E. coli*, the plasmids P<sub>BLA</sub>::*QRI7*::*SUA5*<sub>(M1)</sub> (pBY346.2) and P<sub>BLA</sub>::*QRI7*::*SUA5*<sub>(M10)</sub> (pBY347.6) were tested for complementation of the P<sub>TET</sub>::*tsaC* strain. In this strain background, both plasmids allowed for growth in the absence of aTc, establishing that both *SUA5*<sub>(M1)</sub> and *SUA5*<sub>(M10)</sub> are functional in *E. coli* (Fig. 5D).

## DISCUSSION

In eukaryotes, tRNA modification enzymes targeted to cytoplasm and organelles often are encoded by a single nuclear gene. The dual targeting of the modification enzymes has been observed for several universally conserved tRNA modifications. Six of the eight yeast pseudouridylation enzymes as well as Mod5p, the enzyme responsible for the insertion of N6-isopentenyladenosine (i<sup>6</sup>A<sub>37</sub>), are targeted to both the cytoplasm and the mitochondria (31, 32). Similarly, Trm5p, a tRNA (guanine-N1-)-methyltransferase that modifies mi-



**FIG 5** Qri7 and TsaC are not able to function together *in vivo*, while Qri7 and Sua5 are able to sustain growth. (A) Dilution drops of the *E. coli* P<sub>TET</sub>::*tsaD*(pBAD24) strain and derivatives expressing *QRI7* did not grow in the absence of aTc (required for induction of P<sub>TET</sub>) and the presence of arabinose. When *SUA5* and *QRI7* are expressed as an operon under the P<sub>BAD</sub> promoter, complementation was observed, suggesting that Qri7 and TsaC<sub>Ec</sub> are not able to function together *in vivo*, while Qri7 and Sua5 are at least able to sustain partial growth. The *E. coli* P<sub>TET</sub>::*tsaC*(P<sub>BAD</sub>::*QRI7*) strain does not grow without aTc, while the P<sub>BAD</sub>::*SUA5*::*QRI7* strain can grow without aTc, validating the function of the operon. (B) Diagram of construction of inducible strains and plasmids. (C) Direct transformation of low-copy-number plasmids with constitutive expression from P<sub>BLA</sub> into the P<sub>TET</sub>::*tsaD* strain and plating without aTc. The Qri7::Sua5<sub>(M1)</sub> or Qri7::Sua5<sub>(M10)</sub> synthetic operons do not sustain growth in the absence of aTc. Providing *tsaD* in *trans* using the same setup allows for growth. (D) Direct transformation of low-copy-number plasmids with constitutive expression from P<sub>BLA</sub> into the P<sub>TET</sub>::*tsaC* strain and plating without aTc. Sua5 translated from either the first or second AUG can support the growth of *E. coli* in the absence of aTc.

tochondrial tRNA and also methylates guanosine at position 37 (m<sup>1</sup>G<sub>37</sub>) while tRNAs are in the nucleus, has a dual localization. It has a long form found exclusively in the mitochondria and an N-terminally truncated form (translated from a second internal, in-frame AUG start site) found exclusively in the nucleus (22, 33, 34). The Sua5-GFP data presented here strongly suggest a similar scenario for Sua5p. The nucleotide context surrounding the two AUGs is in a nonoptimum Kozak consensus, with a U at both -3 and +4 for the first AUG (in yeast, a U at -3 reduces expression 2-fold [35]) and a stronger Kozak sequence for the second AUG, with a G at -3 and A

at +4 (Fig. 2A). The presence of two in-frame AUGs at the N terminus of the TsaC/Sua5 proteins is conserved in *Arabidopsis thaliana* and *Homo sapiens* and in all other eukaryotic genomes sequenced to date (see Fig. S4 in the supplemental material), suggesting this dual-targeting mechanism is conserved. In plants, TsaC must be targeted to both mitochondria and chloroplasts in addition to being localized to the cytoplasm, as only one copy of TsaC, located in the nuclear genome, is found in all plant genomes analyzed. This prediction has yet to be experimentally validated.

We reproduced the results reported by Wan et al. showing that



t<sup>6</sup>A-modified tRNA can be formed *in vitro* by joint action of Sua5p and Qri7p in an ATP-dependent fashion (17). The maximum amount of modified tRNA was 16% for our assay conditions; this is significant, but it is not as high as the amount reported by Wan and colleagues (80% of modified tRNA). However, the tRNA substrate we used was a hypomodified tRNA which was overexpressed in *E. coli*. Unfortunately, we do not know the proportion of tRNAs lacking t<sup>6</sup>A modification. The examination of kinetics of our reaction revealed a comparable maximal rate of modification: 0.7% modified tRNA/min versus 0.6% modified tRNA/min for reaction reported by Wan et al. (as judged from Fig. 2G of Wan et al.; also see Fig. S4 in the supplemental material). However, only part of the tRNA put into our reaction can be modified (see Materials and Methods; also see Fig. S5); therefore, our *in vitro* reaction performs equally well or better than that reported by Wan and colleagues. The amount of modified tRNA increased in linear fashion for up to 30 min of incubation, but then the rate decreases, perhaps due to the instability of the proteins. In addition, we observed a similar maximal rate for the cytoplasmic system composed of Sua5p and KEOPS proteins (0.6% modified tRNA/min), indicating that both mitochondrial and cytoplasmic systems perform equally well *in vitro* (see Fig. S5). This observation is in line with the *in vivo* data that demonstrated Sua5p and Qri7p can fully restore t<sup>6</sup>A levels in *kae1Δ* mutant cells of *S. cerevisiae* (17).

We observed that the heterologous system of TsaC and Qri7p performs well *in vitro* (80% of t<sup>6</sup>A formed compared to the control) (Fig. 4B) but does not complement the TsaD mutant *in vivo*. These data indicate that the TC-AMP intermediate produced by TsaC can be captured by Qri7p *in vitro* but not *in vivo*. It is likely that under *in vivo* conditions, the TC-AMP intermediate can be sequestered by other molecules in the crowded interior of the cell (such as nucleotide-binding proteins) or be destroyed before Qri7p captures it. Therefore, our data suggest that the normal t<sup>6</sup>A synthesis in the cell requires that the TC-AMP intermediate be delivered to the Kae1p/TsaD/Qri7p active site. Such a delivery mechanism could be achieved through direct contact between TsaC/Sua5p and Kae1p/TsaD/Qri7p, followed by the channeling of TC-AMP from TsaC/Sua5p into the active site of Kae1p/TsaD/Qri7p. This hypothesis is strongly supported by our data showing that Qri7p can complement the TsaD mutant *in vivo* only when Sua5p also is present, although TsaC is functional and present in the cell. Further support for such interpretation of our data comes from the structural work obtained for the protein TobZ (36). TobZ is highly relevant to t<sup>6</sup>A biosynthesis because it is composed of a TsaC-like domain and Kae1-like domain. Parthier and colleagues showed that the TsaC-like domain catalyzes the formation of the intermediate carbamoyladenylate that is then channeled to the Kae1-like domain, the site of carbamoyl transfer (36). Since both TsaC/Sua5 and TsaD/Kae1/Qri7 families are the constant part of all t<sup>6</sup>A-forming systems, it is likely that all systems operate by channeling of TC-AMP. In line with this prediction, stable interaction between TsaC and TsaD has been reported (10); however, global interactome data for Sua5p did not identify Kae1p or Qri7p proteins as interactants, suggesting that these proteins are engaged in transient interaction (37). Further experimental work is needed in order to test this prediction.

We demonstrated that cells containing only a cytoplasmic version of Sua5p presented a mitochondrial defect, since these cells were unable to grow on the nonfermentable carbon source glycerol. The same phenotype was observed for *qri7Δ* in *S. cerevisiae*

(17), indicating that t<sup>6</sup>A modification is required for the normal functioning of mitochondria. Our data also indicate that TC-AMP produced in the cytoplasm is not transported (in sufficient amounts) to the mitochondrial matrix, which is in line with the unstable nature of this intermediate.

In *S. cerevisiae*, Qri7p can fully rescue t<sup>6</sup>A synthesis in the *kae1Δ* background, but it can only partially complement a telomere-length defect, indicating that KEOPS proteins have one or more other functions in the cell (17). We made a similar observation in *E. coli* cells, since the cells relying only on Qri7/Sua5 for t<sup>6</sup>A synthesis are never as healthy as cells expressing TsaD. Therefore, our data suggest that, as in bacteria, TsaD or a TsaDEB complex has functions in the cell that cannot be replaced by Qri7p, explaining the observed partial complementation. Interestingly, genomic context data implicate TsaDEB in the synthesis of peptidoglycan in bacteria (38). We are pursuing this hypothesis with additional genetic experiments.

The Sua5p localization and phenotype data presented here, in combination with the *in vitro* data published previously (17), which were reproduced in the current study, firmly establish that, in yeast, Sua5p and Qri7p colocalize to the mitochondria and are the only proteins required to introduce t<sup>6</sup>A modifications to mitochondrial tRNAs, and Sua5p must localize to the mitochondria for growth on the nonfermentable carbon source glycerol. This implies that TC-AMP is not transported from the cytoplasm in sufficient amounts for mitochondrial function and that TC-AMP must be produced in this organelle.

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